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Effects of Low Subchronic Doses of Methoxychlor on the Rat Hypothalamic-Pituitary Reproductive Axis

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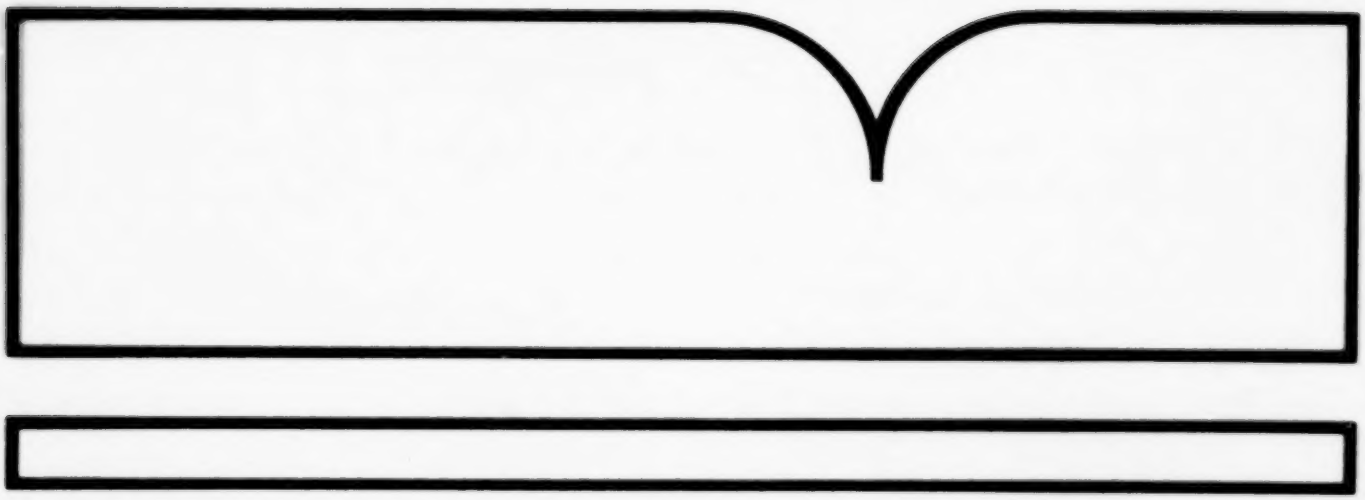

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16. ABSTRACT

The pesticide methoxychlor (MXC) is known to possess a weak estrogenic action and has been found to have a number of toxic effects upon the rodent reproduction system, primarily at the gonadal level. The purpose of this study was to explore the influence of MXC upon the pituitary and hypothalamic components of the male reproductive system at dose levels that were without detectable testicular effects. At 21 days, male Long-Evans rats were gavaged daily with 25 or 50 mg/kg MXC in corn oil. Controls received vehicle only. After 8 weeks of dosing, no significant changes were seen in serum LH, FSH or prolactin, nor in the pituitary concentrations of LH or FSH. Pituitary prolactin was elevated for both doses, and pituitary fragments perifused in vitro released more prolactin than controls. The concentration of gonadotropin-releasing hormone (GnRH) was higher in the mediobasal hypothalamus, but only for the 50 mg/kg group. At this dose, there was a corresponding increase in the KCL-stimulated release of GnRH. The data suggest that previously reported reproductive effects of MXC may be mediated, at least in part, through an elevation in prolactin concentration and release, which in turn is able to influence hypothalamic levels in GnRH. This prolactinemic effect may well represent an early component of the adverse action of MXC upon the reproductive system.

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Effects of Low Subchronic Doses of Methoxychlor on the Rat Hypothalamic–Pituitary Reproductive Axis[1]

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Effects of Low Subchronic Doses of Methoxychlor on the Rat Hypothalamic–Pituitary Reproductive Axis. GOLDMAN, J. M., COOPER, R. L., REHNBERG, G. L., HEIN, J. F., MCELROY, W. K., AND GRAY, L. E., JR. (1986). *Toxicol. Appl. Pharmacol.* **86,** 474–483. The pesticide methoxychlor (MXC) is known to possess a weak estrogenic action and has been found to have a number of toxic effects on the rodent reproductive system, primarily at the gonadal level. The purpose of this study was to explore the influence of MXC on the pituitary and hypothalamic components of the male reproductive system at dose levels that were without detectable testicular effects. At 21 days, male Long–Evans rats were gavaged daily with 25 or 50 mg/kg MXC in corn oil. Controls received vehicle only. After 8 weeks of dosing, no significant changes were seen in serum LH, FSH, or prolactin, nor in the pituitary concentrations of LH or FSH. Pituitary prolactin was elevated for both doses, and pituitary fragments perifused *in vitro* released more prolactin than did controls. The concentration of gonadotropin-releasing hormone (GnRH) was higher in the mediobasal hypothalamus, but only for the 50-mg/kg group. At this dose, there was a corresponding increase in the KCl-stimulated release of GnRH. The data suggest that previously reported reproductive effects of MXC may be mediated, at least in part, through an elevation in prolactin concentration and release, which in turn is able to influence hypothalamic levels of GnRH. This prolactinemic effect may well represent an early component of the adverse action of MXC on the reproductive system. © 1986 Academic Press, Inc.

While the pesticide methoxychlor (MXC) is structurally related to DDT, it is much less toxic and has a relatively short half-life in mammals (Kapoor *et al.*, 1970). It does, however, have a number of adverse effects on the reproductive system. MXC has been observed to accelerate vaginal opening in immature female rat pups and to decrease reproductive performance in these animals as adults (Harris *et al.*, 1974; Gray *et al.*, 1985), an effect which may be associated, at least in part, with atresia of the ovarian follicles (Bal, 1984). In immature male rats it can delay the age of preputial separation (Gray *et al.*, 1985), and in the adult it can decrease testicular weight, arrest spermiogenesis, and cause abnormal spermatocytogenesis and atrophy of the ventral prostate and seminal vesicles (Tullner and Edgcomb, 1962; Gray *et al.*, 1985). MXC has been reported to suppress food intake (Harris *et al.*, 1974) and in other respects behave as a weak estrogen, which is consistent with the above reproductive effects. This estrogenic action does not ap-

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pear to be attributable primarily to MXC itself, but to the generation of a mono-phenol metabolite (Ousterhout *et al.*, 1981). There is an inhibitory effect on estradiol binding to rat uterine cytosol *in vitro* (Bulger *et al.*, 1978) which implies that the mode of action corresponds to that of the nonsteroidal estrogen, diethylstilbestrol (DES), although the inhibition by this metabolite is considerably less potent on an equimolar basis. DES is known to have marked effects on the hypothalamic–pituitary–gonadal axis that include reductions in serum-luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone, and elevations in serum prolactin (Prl) and hypothalamic gonadotropin-releasing hormone (GnRH) (Bartke *et al.*, 1984; Jackson and Franklin, 1984; Cooper *et al.*, 1985).

Subchronic oral dosing regimens of 100 or 200 mg MXC/kg body wt/day have been found to cause a variety of testicular changes, including decrements in the weights of whole testis (6 and 18%, respectively), cauda epididymus (18 and 41%), seminal vesicles (39 and 71%), and a drop in serum testosterone (79 and 96%) and cauda sperm count (Gray *et al.*, submitted). Additionally, it was found that these doses of methoxychlor resulted in decreased pituitary weight. The effects of DES on brain and pituitary indicate that at least some of its effects on testicular tissue may not necessarily be direct, but mediated through alterations in the blood-borne signals regulating testis function. Similarly, the fact that MXC caused changes in pituitary weight would suggest that this compound is exerting some effect on pituitary hormone synthesis and secretion. Shi *et al.* (1986) have recently reported that shifts in serum testosterone levels are able to alter the concentrations of pituitary hormones. Consequently, it was decided to investigate the effects of methoxychlor on the endocrine activity of the hypothalamic–pituitary axis, especially given the sensitivity of these pituitary measurements to DES treatment (Cooper *et al.*, 1985) and the similarities of the MXC-induced reproductive changes to those observed following DES administration. To minimize the possibility that alterations at the gonadal level are involved in any observed endocrine changes in the hypothalamic–pituitary reproductive axis, lower MXC doses of 50 and 25 mg/kg/day were selected for the present study, as they have not been found to affect common testicular measures under these conditions.

METHODS

Ninety-day-old pregnant Long–Evans hooded rats were obtained from Charles River Breeding Laboratory (Portage, Mich.) on Day 13 of gestation. They were maintained on a 12-hr:12-hr photoperiod (lights on, 0600 hr) in individual clear plastic cages with laboratory-grade pine shavings as bedding. Food and water were available *ad libitum*. On postpartum Day 6, the litters were culled to eight animals (equally divided between sexes). The pups were weaned at 21 days and, after weighing, were assigned to treatment groups having equal means and variances in body weight. Weights were subsequently recorded twice weekly for the duration of the study. Based on preliminary work with a dose range of 25 to 200 mg/kg body wt/day, the administration of MXC was begun that day in males by gavage for the following three groups: (1) sham control receiving corn oil vehicle only, (2) 25 mg MXC/kg/day (Sigma, lot M-1501, 90% purity) in corn oil, and (3) 50 mg/kg/day in corn oil. The animals were initially given 0.5 μl/g body wt, a volume which was continued until they reached 200 g. The quantity was then increased to 2.5 μl/g until the time of sacrifice. This protocol is consistent with one currently in use (Gray *et al.*, 1985) for the assessment of effects of potential toxicants on pubertal reproductive development.

Dosing was continued on a daily basis for 56 consecutive days, after which the rats were rapidly sacrificed by decapitation (0900–1030 hr). Trunk blood was collected for analysis of luteinizing hormone, follicle-stimulating hormone, prolactin, and testosterone. Within 90 sec of sacrifice, the pituitaries were removed and the anterior lobes weighed and hemisected. A single half was then frozen for later hormonal determinations. To assess potentially more subtle effects of MXC on hormonal release, fragments of the other hemisected anterior pituitary from control and experimental groups were individually placed in cold culture medium for *in vitro* perifusion (described below). At the same time, the brains from these three groups were promptly removed and the mediobasal hypothalmus (MBH) dissected out on ice. This area was defined rostrally by a cut at the ventral margin of the optic chiasm, caudally by a cut just behind the infundibulum, laterally by two cuts 1 mm medial to the hypotha-

lamic sulci, and dorsally by a section at the ventral margin of the anterior commissure. A number of MBH fragments were promptly placed in culture medium for the *in vitro* assessment of gonadotropin-releasing hormone secretion by perifusion (see below). The remainder were frozen for tissue GnRH analysis.

Perifusion. The standard culture medium for both pituitary and MBH tissue was sterile Hepes buffered (10 mM, pH 7.4) medium 199 (K. C. Biological) containing 0.3% bovine serum albumin (Sigma, fraction V). Each hemipituitary to be perifused was further sectioned into 1-mm^3 pieces and placed between presoaked Pyrex glass wool plugs in a 500-μl chamber constructed from a disposable 3-cc syringe. Buffer was pumped (Manostat Jr. peristaltic pump) through the chamber at a flow rate of 160 μl/min and the chamber effluent collected in glass 12 × 75-mm tubes at 9-min intervals, before being frozen at −65°C.

Two hours of perifusion allowed the pituitaries to reach a level of baseline hormonal release. At the end of this period, the fragments were subjected to constant stimulation with GnRH (Boehringer–Mannheim, 100 ng/ml in medium 199/BSA). The challenge was then continued for an additional 2.5 hr. Fifty-four minutes before the conclusion of the procedure, the pituitary fragments were stimulated for 9 min with 60 mM potassium chloride (KCl, isosmotic in medium 199/BSA). Immediately after the final collection of effluent, the tissues were removed from the chambers, sonicated (Fisher 300 sonic dismembrator), and frozen at −65°C.

For the perifusion of MBH tissue, the standard culture medium (described above) was gassed with 95% O_2/5% CO_2 and supplemented with bacitracin (50 μM, Sigma) in order to inhibit the metabolic degradation of GnRH by tissue peptidases (McKelvy *et al.*, 1976).

At sacrifice, excised MBH fragments were quickly weighed, sectioned at the midline, and placed in perifusion chambers (between glass wool plugs) together with another MBH fragment from that treatment group (mean weights of chambered MBH tissue: 58.3 ± 1.7 mg). These chambers were constructed from disposable 1-cc insulin syringes and had an internal volume of 200 μl. The flow rate was adjusted to 100 μl/min over the initial 15 min, after which time effluents were collected at 7.5-min intervals. Following a 50-min equilibrium period, the tissue was challenged with 3 pulses of 60 mM KCl (final concentration, isosmotic in medium 199/BSA). The elevated K^+ medium was introduced through a closable injection port into the bottom of the chamber, below the lower glass wool plug. For such general non-receptor-mediated depolarizations, KCl concentrations of 50–60 mM have been commonly used in studies of transmitter and hormonal release *in vitro* to investigate the capacity of perifused hypothalamic tissue to respond to stimulation (e.g., Brabant et al., 1985; Becker and Ramirez, 1980; Drouva et al., 1981). Moreover, with brief periods of stimulation, these pulses do not appear to affect adversely the fine structure of nerve endings within this region (Brabant *et al.*, 1985; Zamora and Ramirez, 1983).

Upon collection, the effluents in each tube were promptly frozen at −65°C for subsequent GnRH measurement. After perifusion, the MBH fragments within the chambers were gently blotted and weighed, before being diluted 1:10 (w/v) in cold 0.1 N $HClO_4$ (Wilkes and Yen, 1981) and sonicated.

Hormonal assays. All hormones were analyzed by radioimmunoassay. The concentrations of LH, FSH, and Prl were determined with the following materials supplied by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases: iodination prep.—I-6 (LH), I-6 (FSH), I-5 (Prl); reference prep.—RP-2 (LH), RP-2 (FSH), RP-3 (Prl); antisera—S-8 (LH), S-11 (FSH), S-9 (Prl). Using chloramine-T (Sigma), the purified iodination preparations were radiolabeled with ^{125}I obtained as carrier-free Na ^{125}I in NaOH (New England Nuclear) by a modification of the method of Greenwood *et al.* (1963). Each labeled hormone was separated from unreacted iodide on a Biogel P-60 (Bio-Rad Laboratories) column with potassium phosphate buffer (50 mM, pH 7.5) as the eluent. The assays themselves were carried out according to recommendations accompanying each kit, and all used goat anti-rabbit γ-globulin (Calbiochem) as second antibody. The assay sensitivities for LH and FSH were increased by a 24-hr coincubation of sample and first antibody prior to the addition of labeled hormone for an additional 24 hr. The respective sensitivities and inter- and intrassay variances for the three pituitary hormones were 15 pg/tube, 7.7 and 8.1% (LH), 3 pg/tube, 8.3 and 12.4% (FSH), and 32 pg/tube, 1.9 and 9.0% (Prl).

Serum testosterone was analyzed with radioimmunoassay kits purchased from Diagnostic Products (Los Angeles). Interassay and intraasay variances were 4.0 and 2.9%, respectively. GnRH as measured using antiserum No. 428 kindly provided by Dr. P. M. Conn (University of Iowa). It recognized the C-terminal of GnRH and does not cross-react in radioimmunoassay with a variety of other peptides, including TRH, somatostatin, vasopressin, GHRH, CRF (all ≤ 0.001%) or des-Gly^{10} GnRH (≤0.01%). It was used at a tube dilution of 1:28,000. Synthetic GnRH (Boehringer–Mannheim) was iodinated by a modification of the chloramine-T procedure of Greenwood *et al.* (1963) and purified by ion-exchange chromatography on a carboxymethylcellulose (Sigma) column, with ammonium acetate (2 and 60 mM, pH 4.5) as eluent (Heber *et al.*, 1978). The assay was similar to that described by Nett and Niswender (1979), using second antibody to separate free hormone from bound. As with LH and FSH, the sensitivity was optimized (to between 0.7 and 1.1 pg/tube) by a prior 24-hr coincubation of sample and first antibody. The inter- and intraassay variances were 12.1 and 9.8%, respectively.

Statistical treatment. Significant effects among groups were tested by analysis of variance under the General Lin-

TABLE 1

PITUITARY AND TESTICULAR WEIGHTS AND SERUM HORMONAL CONCENTRATIONS (MEAN GROUP VALUES ± SEM)

	Control ($n = 7$)	25 mg MXC/kg ($n = 8$)	50 mg MXC/kg ($n = 8$)
Pituitary weight (mg)	10.17 ± 0.57	9.98 ± 0.49	9.34 ± 0.35
Serum LH (ng/ml)	0.54 ± 0.05	0.56 ± 0.05	0.50 ± 0.05
Serum FSH (ng/ml)	7.90 ± 0.42	7.72 ± 0.42	8.70 ± 0.43
Serum Prl (ng/ml)	3.64 ± 0.83	6.03 ± 1.46	8.47 ± 2.78
Serum testosterone (ng/ml)	3.27 ± 0.48	3.39 ± 0.78	3.98 ± 0.94
Testis weight (g)	1.72 ± 0.06	1.58 ± 0.05	1.57 ± 0.07

ear Models procedure of the Statistical Analysis System (SAS) package, with the α level set at 0.05. For perifusion, data were compared at each time point for differences among group means. Significant effects were then compared further among groups by t test (two-tailed).

RESULTS

Pituitary

The influence of 25 and 50 mg MXC/kg on anterior pituitary and testicular weights and

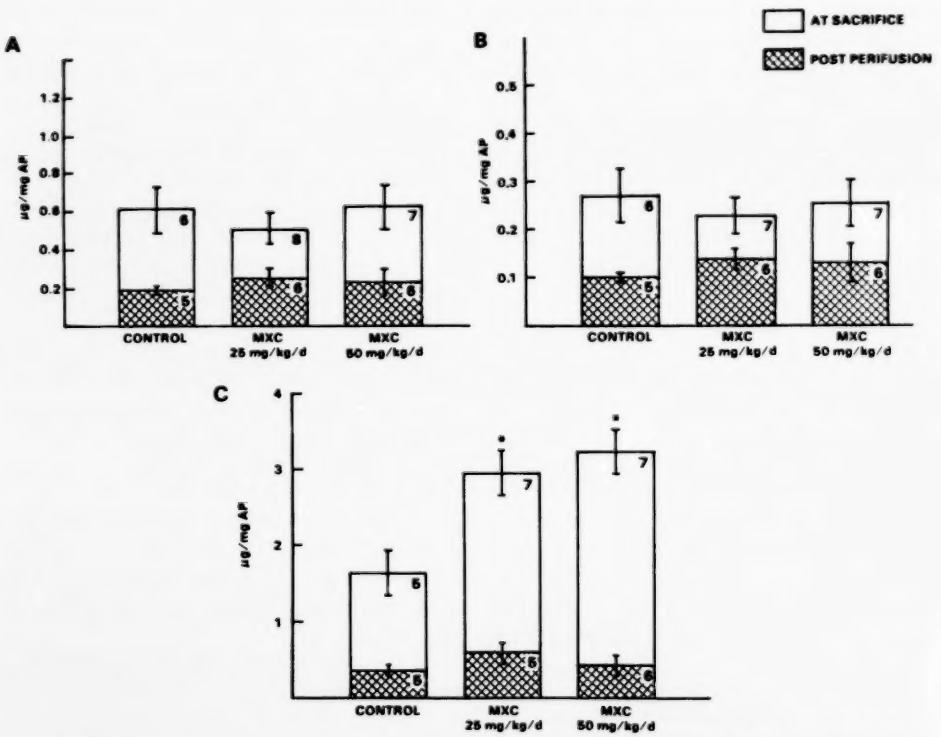

FIG. 1. Mean pituitary hormonal concentrations at the time of sacrifice and following the completion of perifusion. (A) LH, (B) FSH, (C) prolactin. Values are in µg/mg anterior pituitary ± SEM. Number of animals per group is listed within each column. *$p \leq 0.05$ as compared with controls.

on serum LH, FSH, prolactin, and testosterone is shown in Table 1. Under these conditions there were no significant effects on these tissue weights nor on LH, FSH, and testosterone concentrations. Serum prolactin did appear to be elevated somewhat for both experimental groups, although in neither case did the statistical probability level reach 0.05. Figure 1 presents the anterior pituitary concentrations of each hormone both at sacrifice and after the cessation of perifusion. No statistical differences were seen for LH or FSH at either time point. The values for prolactin, however, were elevated at sacrifice in both treatment groups ($p \leq 0.05$). No such effects were observed in the postperifusion analysis.

The release *in vitro* of both LH and FSH (Fig. 2) is consistent with the findings for the serum and pituitary concentrations of these hormones. No differences between the control and MXC groups were found for the baseline hormonal secretion or the stimulated release in response to either GnRH or KCl. By contrast, both the nonstimulated and KCl-challenged release of prolactin were significantly elevated for the 25- and 50-mg/kg/day MXC groups (Fig. 3). A dose–response relationship was present for nonstimulated prolactin secretion at all time points during perifusion.

Mediobasal Hypothalamus

The concentrations of GnRH within the mediobasal hypothalamus at sacrifice are shown in Fig. 4. In comparison with controls, the values for the 50-mg/kg/day group were significantly increased ($p \leq 0.05$). The lower MXC dose was without effect. While no alterations were found among the groups in baseline secretion, this change in the 50-mg/kg/day group was mirrored by the influence of this dose on KCl-stimulated GnRH release (Fig. 5).

DISCUSSION

After 8 weeks of daily gavage with MXC, neither 25 nor 50 mg/kg/day was observed to alter anterior pituitary weight. Higher doses, however, did cause a decrease in pituitary weight which became statistically significant at a dose of 200 mg/kg/day (Gray *et al.*, 1985). This effect of MXC appears to be in contrast to the influence of DES and estradiol on Long–Evans rats (Cooper *et al.*, 1985), which shows a positive correlation between dose and anterior pituitary weight. While each of these compounds has been found to increase prolactin levels, this differential effect on pituitary weight suggests that the actions of these estrogenic substances are not identical.

The results of the present study also show that under the protocol employed there are elevations in the pituitary concentration and release of prolactin. These changes occur at doses of MXC which are not found to influence statistically the serum levels of this hormone, indicating that serum measures may, in some instances, be relatively insensitive indices (possibly due to alterations in hormonal clearance, etc.) of the endocrine effects of a potential toxicant. This alteration in pituitary release of prolactin agrees with those estrogen-associated hyperprolactinemic effects reported for both estradiol (Terry *et al.*, 1985) and DES (Bartke *et al.*, 1984; Cooper *et al.*, 1985; Lloyd, 1983). And, since there is strong evidence for a relationship between elevated estrogen levels and tumor activity (e.g., Brawer and Sonnenschein, 1975; Nakagawa *et al.*, 1980; Cooper, 1983), this effect may be involved in the previously reported tumorigenic response to MXC (Radomski *et al.*, 1965; Reuber, 1980). The finding that no differences exist between groups in the postperifusion tissue analysis indicates that the differential concentrations of pituitary prolactin at sacrifice represent releasable stores which are largely depleted over the course of perifusion.

In contrast to these effects of MXC on prolactin, neither the concentrations nor the release of LH or FSH were affected. No differences were seen in serum or pituitary levels, and the baseline and stimulated release *in vitro* (with GnRH or KCl) was

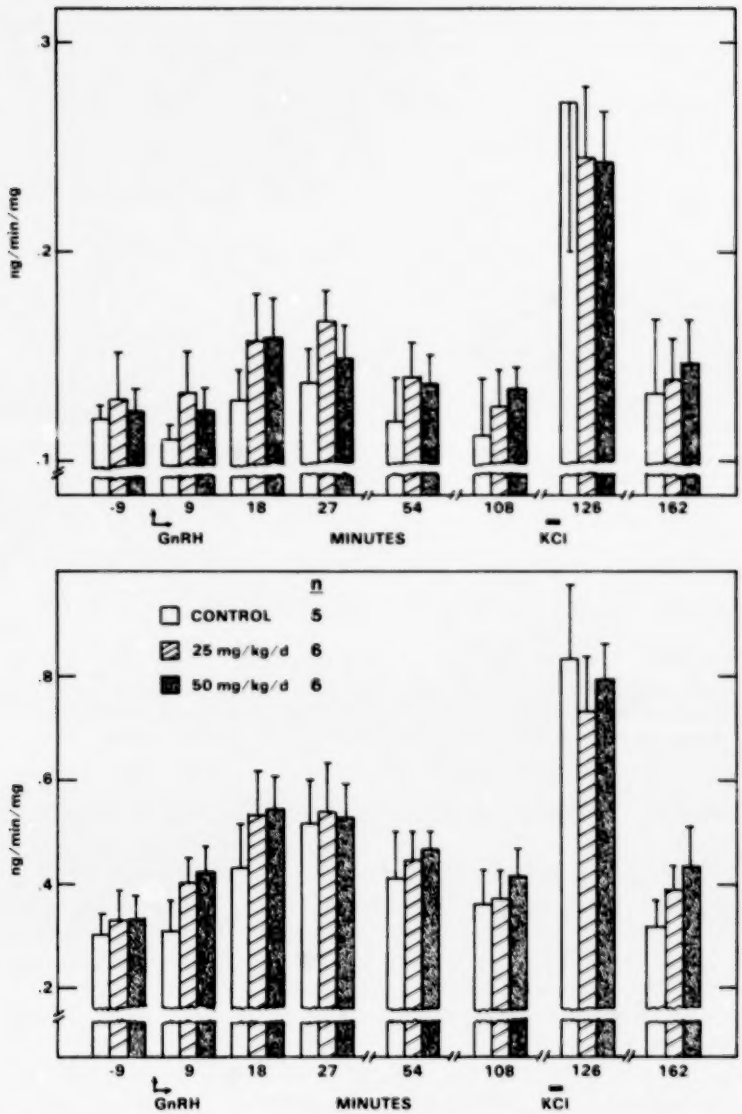

FIG. 2. Release of LH (upper) and FSH (lower) by perifused hemipituitary fragments. Vertical arrow on horizontal axis identifies point (0 time) at which continuous GnRH challenge (100 ng/ml) was begun. The 9-min KCl stimulus (60 mM) is represented by the short bar under the right portion of the axis. Hormonal release is in ng/min/mg anterior pituitary ± SEM.

comparable with controls. In fact even with MXC doses of 100 and 200 mg/kg, no significant alterations were present in serum LH (−10 and −15%, respectively, against controls) nor FSH (+26 and +7%). These results are consistent with the absence of any significant shifts in testicular measures, such as serum testosterone and testis weight, which

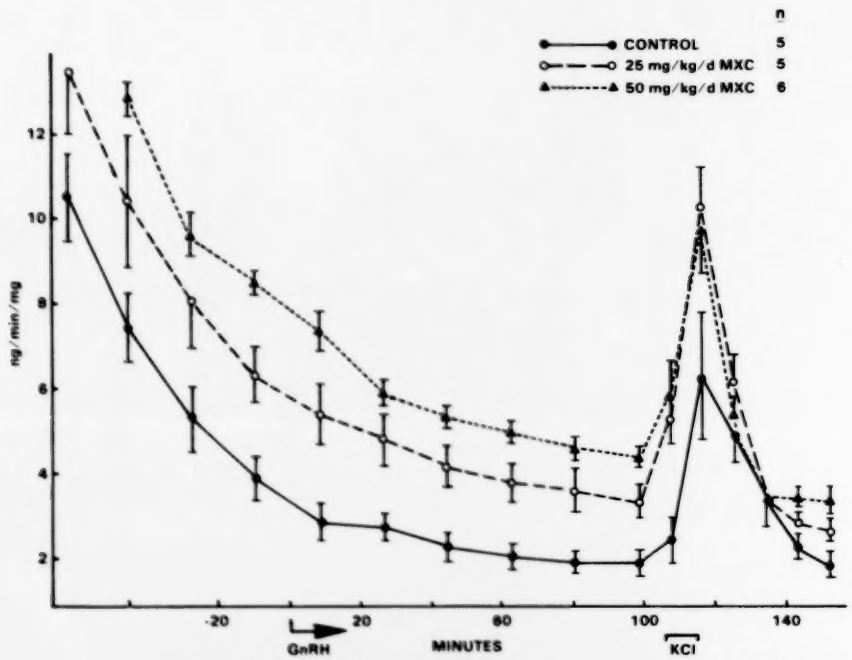

FIG. 3. Release of prolactin by perifused hemipituitary fragments. The arrow depicts point (0 time) at which continuous GnRH challenge was begun. The 9-min KCl stimulus is represented by the short bar under the right portion of the horizontal axis. Release is in ng/min/mg anterior pituitary ± SEM.

are directly affected by changes in gonadotropin secretion.

Diethylstilbestrol has been reported to increase the concentrtions of mediobasal hypothalamic GnRH through an inhibitory influence on GnRH release (Bartke *et al.*, 1984). The change appears to be related to an elevation in prolactin which feeds back upon the hypothalamus to in turn affect the dopaminergic regulation of both prolactin and GnRH secretion (Ojeda *et al.*, 1974; Lookingland and Moore, 1984). Thus, the observed alterations in GnRH concentration in the 50-mg/kg/day MXC group may involve some local effect of altered prolactin levels induced by methoxychlor. In the present study, the increase in MBH GnRH concentration was not accompanied by a suppression in the baseline secretion of GnRH *in vitro*. Moreover, the augmented response to KCl reflected the elevation in the MBH value. This is in contrast to the decrease in the stimulated release of GnRH following DES administration (Bartke *et al.*, 1984). The difference may be attributable to the nonspecific nature of the KCl stimulus, which represents a generalized non-receptor-mediated depolarization, bypassing any effect on the transmitter systems controlling GnRH liberation. The absence of an MXC-induced alteration in the baseline GnRH release is, nevertheless, consistent with the lack of influence of these pesticide doses on the gonadotropin measures.

The results of the present study indicate that low subchronic doses of MXC administered by gavage cause an increase in both the pituitary prolactin concentration and secretion of this hormone *in vitro*. This change is not reflected by a significant shift in serum prolactin and is unlikely to involve any feed-

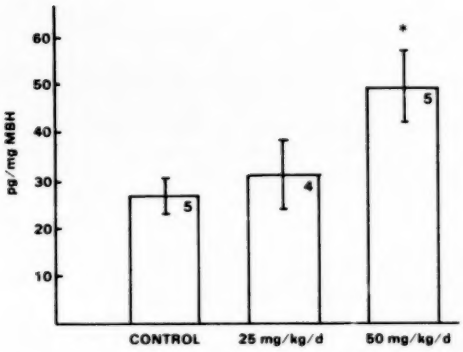

FIG. 4. Mean GnRH concentrations within the mediobasal hypothalamus at sacrifice. Values are in pg/mg MBH ± SEM. Number of animals per group is listed within each column. $^{*}p \leq 0.05$ as compared with controls.

back effects at the testicular level, since no gonadal changes have been seen at these MXC doses. A local feedback of increased prolactin on the hypothalamus may be involved in an elevation in the concentration of GnRH within the MBH in response to the 50-mg/kg/day dose. While no alterations were seen in the baseline levels of GnRH release, there was a significant increase in GnRH release in response to a series of KCl pulses. These effects of MXC on prolactin are in general agreement with the reported estrogenic action of MXC. However, the differences between the effects of estradiol/DES and MXC on anterior pituitary weight indicate that there is some divergence in the overall effects of these substances on the hypothalamic–pituitary endocrine axis.

The changes in prolactin and GnRH observed in the present study indicate that such measures are sensitive predictors of the emerging adverse effects of weak estrogenic agents on the reproductive system and, as such, represent early components of the action of MXC on the hypothalamic–pituitary–testicular axis. While no toxic effects on the testes were observed under the present dosing protocol, hyperprolactinemia induced with pituitary isografts or subcutaneous prolactin

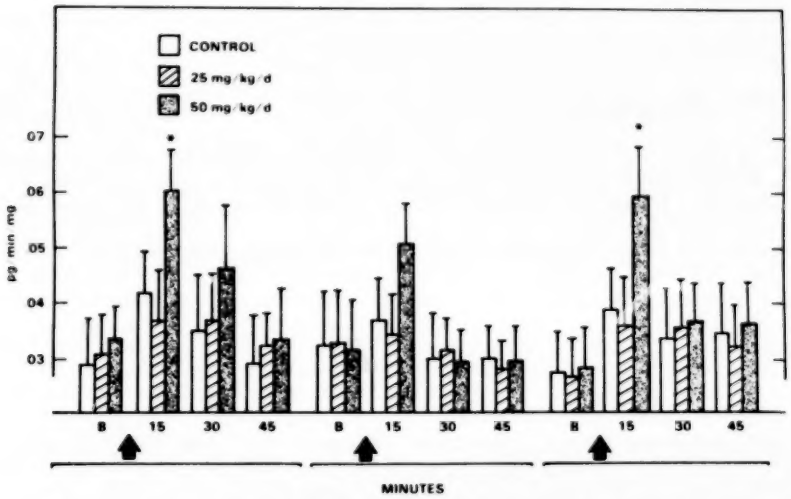

FIG. 5. GnRH release *in vitro* from perifused fragments of MBH. The horizontal axis is segmented into three challenge periods which are further divided into a prechallenge baseline release (B) and succeeding 15-min postchallenge intervals. Arrows indicate the point at which KCl pulse (60 mM) was administered. Values are in pg/min/mg MBH ± SEM. $^{*}p \leq 0.05$ as compared with controls for that time interval.

injections has been found to alter a number of testicular parameters, including seminal vesicle and prostate weights (Nonomura *et al.*, 1985) and the availability of LH/hCG binding sites in rat testis (Leydig cell preparations (Morris and Saxena, 1980). Since the elevations in serum prolactin, although statistically nonsignificant, may be biologically important, it is possible that a continued administration of this pesticide would eventually induce those alterations in testicular weight, sperm count, and testosterone levels seen with higher doses. What is clear is that such neuroendocrine alterations need to be examined more closely for both young and adult periods of treatment and under longer dosing conditions in order to determine their significance to a overall reproductive effect.

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